Role of purine N-3 in the biologic activities of poly(A) and poly(I).

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ABSTRACT

Poly(c^3A) (poly 3-deazaadenylic acid) and poly(c^3I) (poly 3-deazainosinic acid) differ in biological reactivity from their parent compounds poly(A) and poly(I) and from their 7-deaza counterparts poly(c^7A) and poly(c^7I). Three parameters of biological reactivity were evaluated : (1°) interferon induction, (2°) anti-complement activity, (3°) reverse transcriptase inhibition. Unlike poly(A).poly(U), poly(I).poly(C) and poly(I). poly(br^5C), the mixtures of poly(c^3A) + poly(U), poly(c^3I) + poly(C), and poly(c^3I) + poly(br^5C) failed to elicit an interferon response in "superinduced" primary rabbit kidney cells. Poly(I) and its analogs poly(c^3I) and poly(c^7I) inhibited hemolytic complement activity, whereas poly(A) and its analogs poly(c^3A) and poly(c^7A) failed to do so. Both poly(I) and poly(c^7I), but not poly(c^3I), lost their anti-complement potency when annealed to either poly(C) or poly(A).poly(U). Similarly, poly(I) and poly(c^7I), but not poly(c^3I), suppressed the interferon inducing ability of poly(A).poly(U), suggesting that both poly(I) and poly(c^7I), but not poly(c^3I), added to poly(A).poly(U) to form a triple-helical structure. Poly(I), poly(c^7I) and poly(c^7A) exerted a distinct inhibitory effect on the endogenous RNA directed DNA polymerase (reverse transcriptase) activity of murine leukemia virus, while under the same conditions poly(c^3I) and poly(c^3A) showed little, if any, inhibitory effect.

INTRODUCTION

A major requisite for the interferon inducing activity of homopolyribonucleotide duplexes such as poly(A).poly(U), poly(I).poly(C) and poly(br^5C) is an intact purine ring. Substitution of CH for N-7 in the purine bases of either poly(A).poly(U) or poly(A).poly(rT) led to an almost

complete loss of the interferon inducing ability of both complexes (1). A similar but less pronounced decrease of interferon inducing activity was observed upon substitution of CH for N-7 in the purine strands of poly(I). poly(C) and poly(I).poly(br^{5}C); however, in some assay conditions (primary rabbit kidney cells or human skin fibroblasts "superinduced" with metabolic inhibitors) the N-7 substituted analog poly(c^{7}I).poly(br^{5}C) equalled or even surpassed its parent compound in activity (2,3).

Herein we report on the biological implications of another nuclear modification, substitution of CH for N-3 in the purine ring of both poly(A) and poly(I). The resulting analogs poly(c^{3}A) and poly(c^{3}I) were examined for interferon inducing ability upon proper mixing with poly(U) and poly(C) [or poly(br^{5}C)], respectively. In addition, poly(c^{3}A) and poly(c^{3}I) were analyzed for two other biological potentials : anti-complement activity and inhibition of the RNA directed DNA polymerase (reverse transcriptase) activity of oncornaviruses. Poly(I) is a powerful inhibitor of complement (4 and references cited therein). Poly(I) has also been found to inhibit reverse transcriptase activity (5-8) and, recently, we have shown that introduction of a methylthio substituent at C-2 markedly increases the inhibitory effect of poly(I) on the reverse transcriptase of murine (Moloney) leukemia virus (8). The role of N-3 in the biological activities of poly(I) has now been assessed. To this end, poly(c^{3}I) and its counterpart poly(c^{3}A) were directly compared to the unmodified parent compounds poly(I) and poly(A) in the different bioassay systems. Poly(c^{7}A) and poly(c^{7}I) were also included in these tests to evaluate the relative importance of N-3 as compared to N-7.

MATERIALS AND METHODS

The synthesis and physicochemical characteristics of poly(c^{3}A), poly(c^{7}A), poly(c^{3}I) and poly(c^{7}I) have been described previously (9-12). The synthesis of poly(br^{5}C) (s$_{20,w}$ = 11.5 S) has also been described (2).

Abbreviations : poly(A) : polyadenylic acid; poly(U) : polyuridylic acid; poly(I) : polyinosinic acid; poly(C) : polycytidylic acid; poly(rT) : poly(5-methyluridylic acid); poly(br^{5}C) : poly(5-bromocytidylic acid); poly(c^{3}A) : poly(3-deazaadenylic acid); poly(c^{7}A) : poly(7-deazaadenylic acid); poly(c^{3}I) : poly(3-deazainosinic acid); poly(c^{7}I) : poly(7-deazainosinic acid). MuLV (Moloney) : Moloney strain of murine leukemia virus; PRK : primary rabbit kidney; MEM : Eagle's minimal essential medium.

The following homopolyribonucleotides were purchased from Miles Laboratories (Elkhart, Indiana) : poly(A) ($s_{20,w}$ = 5.5, 8.9 S), poly(U) ($s_{20,w}$ = 6.0, 7.4 S), poly(C) ($s_{20,w}$ = 5.2 S) and poly(I) ($s_{20,w}$ = 4.8 S). The poly(I).poly(C) complex employed in our interferon induction studies was composed of homopolymers purchased from P-L Biochemicals (Milwaukee, Wisconsin) : poly(I) ($s_{20,w}$ = 9.4 S) and poly(C) ($s_{20,w}$ = 10.0 S). All homopolymers were dissolved in 0.1M Tris-HCl, 0.2M NaCl, pH 7.0 at 1 mg/ml and stored at 4°. The homopolymer complexes poly(A).poly(U) and poly(I).poly(C), however, were prepared at 1 mg/ml in PBS (Dulbecco's phosphate buffered saline) and stored at -20°.

Physicochemical studies on the interaction of poly(c^3A) with poly(U) have been reported before (9). Extensive physicochemical characterization of the interaction between poly(c^3I) and poly(C) or poly(br^5C) was not
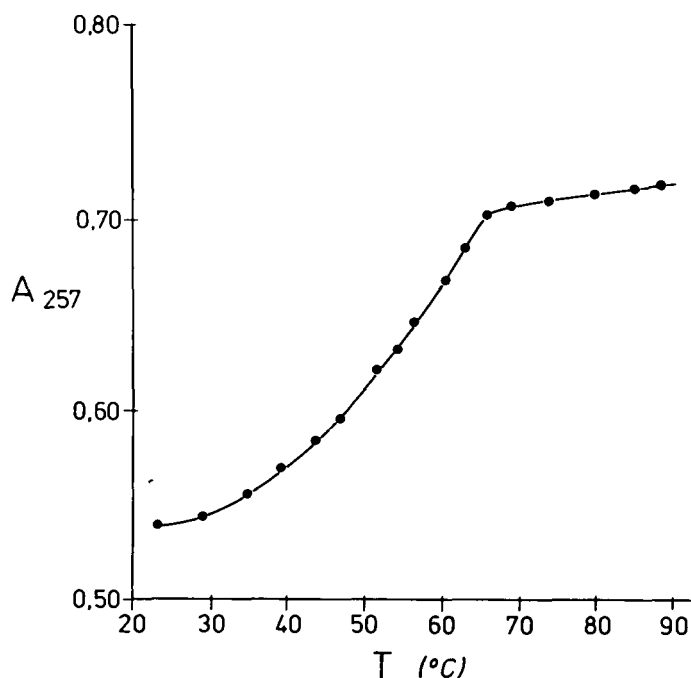

Figure 1. - Melting profile of poly(c^3I).poly(br^5C) (10^{-4}M) in 0.10M NaCl, 0.01M KH_2PO_4, pH 7.2. Same melting behavior was noted at 245, 270, 280 and 290 nm.

possible because of the limiting amounts of poly(c^3I) available. However, the following observations were made :

(1) A solution equimolar in both poly(c^3I) and poly(br^5C) gave a UV spectrum that was hypochromic with regard to the summation spectrum of the two components (assuming no interaction). For instance, at 257 nm [the λmax of poly(c^3I)], the 1:1 poly(c^3I).poly(br^5C) mixture showed 20 % hypochromicity with respect to that expected if no interaction occurred (data not illustrated).

(2) The UV-absorbance temperature profile of the 1:1 poly(c^3I).poly(br^5C) mixture (Fig. 1) showed a broad non-cooperative transition occurring between 30° and 70° with a midpoint (Tm) of 52° (in 0.10M NaCl, 0.01M KH_2PO_4, pH 7.2). Such behavior was not exhibited by either poly(c^3I) or poly(br^5C).

(3) In contrast to the poly(c^3I) + poly(br^5C) mixture, a 1:1 mixture of poly(C) and poly(c^3I) (in 0.10M NaCl, 0.01M KH_2PO_4, pH 7.2) showed no significant transition over a temperature range of 9° to 80°.

Interferon production was measured in PRK (primary rabbit kidney) cells "superinduced" with cycloheximide and actinomycin D (1). Therefore, confluent PRK cell monolayers (in petri dishes) were exposed to the polymer (concentration as indicated in the footnote to Table 1) in 1 ml MEM per petri dish for 1 hour at 37°. The cells were then washed (3x) with MEM, treated with cycloheximide (2 μg/ml in MEM + 3 % calf serum; 2 ml per petri dish) for 3 hours at 37°, washed again, treated with actinomycin D (3 μg/ml in MEM + 3 % calf serum; 2 ml per petri dish) for 30 minutes at 37°, washed again, and further incubated with MEM + 3 % calf serum (4 ml per petri dish) for 20 hours at 37°. The supernatant fluids of the cell cultures were then harvested and titrated for interferon (1).

The methodology for evaluating anti-complement activity has been described previously (4). The methodology for measuring MuLV (Moloney) DNA polymerase (reverse transcriptase) activity has also been described (13,14). Carbopol (a carboxypolymethylene) was included in the reverse transcriptase reaction mixtures to increase the sensitivity of the assay (14).

RESULTS

Interferon induction. In marked contrast with poly(A).poly(U), poly(I).poly(C) and poly(I).poly(br^5C) which induced 1000 to 10,000 units/ml of interferon when exposed to PRK cells at 10 μg duplex per ml, the 1:1

mixtures $poly(c^3A).poly(U)$, $poly(c^3I).poly(C)$ and $poly(c^3I).poly(br^5)$ were devoid of any interferon inducing activity (Table 1). The 1:2 mixture $poly(c^3A).2$ $poly(U)$ was also ineffective, which is not unexpected in view of the well established inefficacy of triple-stranded complexes as inducers of interferon (1,15). When mixed with $poly(A)$, the activity of $poly(c^3A).$ $poly(U)$ raised to the level normally observed with $poly(A).poly(U)$, and, when $poly(c^3I).poly(C)$ or $poly(c^3I).poly(br^5C)$ were mixed with either $poly(I)$ or $poly(c^7I)$, their activity attained the level characteristic of $poly(I).poly(C)$, $poly(I).poly(br^5C)$ or $poly(c^7I).poly(br^5C)$, suggesting that under the experimental conditions employed the initial complexes, if formed, dismutated according to the following reaction schemes :

i) $poly(c^3A).poly(U)$ + $poly(A)$ ⟶ $poly(c^3A)$ + $poly(A).poly(U)$

ii) $poly(c^3I).poly(C)$ + $poly(I)$ ⟶ $poly(c^3I)$ + $poly(I).poly(C)$

iii) $poly(c^3I).poly(br^5C)$ + $poly(I)$ ⟶ $poly(c^3I)$ + $poly(I).poly(br^5C)$

iv) $poly(c^3I).poly(br^5C)$ + $poly(c^7I)$ ⟶ $poly(c^3I)$ + $poly(c^7I).poly(br^5C)$

That $poly(A).poly(U)$, $poly(I).poly(C)$, $poly(I).poly(br^5C)$ and $poly(c^7I).poly(br^5C)$ retained their full interferon inducing capacity in the presence of either $poly(c^3A)$ or $poly(c^3I)$ was ascertained by measuring the interferon inducing properties of these duplexes after they had been mixed with either $poly(c^3A)$ or $poly(c^3I)$ or control medium (MEM). No differences were witnessed (Table 1).

Unlike $poly(c^3A)$, $poly(c^7A)$ suppressed the interferon inducing activity of $poly(A).poly(U)$, and the activity normally observed with $poly(A).$ $poly(U)$ was not attained upon addition of $poly(A)$ to the $poly(c^7A).poly(U)$ mixture. As pointed out before (16), the relatively low interferon titers obtained with the systems $poly(c^7A).poly(U)$ + $poly(A)$ and $poly(A).poly(U)$ + $poly(c^7A)$ appear to be accounted for by the detrimental effect of $poly(c^7A)$ on cellular RNA synthesis (7).

When mixed with $poly(I)$, $poly(A).poly(U)$ loses a significant part of its interferon inducing activity, and the reason for this phenomenon has been determined (17) to be the formation of the triple-helical complex $poly(A).poly(U).poly(I)$. The low interferon inducing activity of the system $poly(A).poly(U)$ + $poly(c^7I)$ (Table 1) suggests that $poly(c^7I)$ may also form a triple-helical complex with $poly(A).poly(U)$. The fact that $poly(c^3I)$ does not suppress the interferon inducing activity of $poly(A).$ $poly(U)$ may be considered as evidence against such triplex formation.

<u>Anti-complement activity</u>. In keeping with previous data (4), $poly(I)$

TABLE 1. INDUCTION OF INTERFERON IN PRK CELLS SUPERINDUCED
WITH CYCLOHEXIMIDE AND ACTINOMYCIN D

Mixture[*]			Interferon titer (units/ml)
Complex[+]		Homopolymer	range
$poly(c^3A).poly(U)$	+	MEM	<10
$poly(c^3A).poly(U)$	+	poly(A)	600 - 3000
$poly(c^3A).2\ poly(U)$	+	MEM	<10
$poly(c^3A).2\ poly(U)$	+	poly(A)	<10
$poly(c^7A).poly(U)$	+	MEM	<10
$poly(c^7A).poly(U)$	+	poly(A)	30 - 300
poly(A).poly(U)	+	MEM	1000 - 3000
poly(A).poly(U)	+	$poly(c^3A)$	600 - 1000
poly(A).poly(U)	+	$poly(c^7A)$	20 - 200
poly(A).poly(U)	+	$poly(c^3I)$	1000 - 1500
poly(A).poly(U)	+	$poly(c^7I)$	30 - 60
poly(A).2 poly(U)	+	MEM	<10
poly(A).2 poly(U)	+	$poly(c^3A)$	<10
$poly(c^3I).poly(C)$	+	MEM	<10
$poly(c^3I).poly(C)$	+	poly(I)	3000 - 10000
$poly(c^3I).poly(br^5C)$	+	MEM	<10
$poly(c^3I).poly(br^5C)$	+	poly(I)	3000
$poly(c^3I).poly(br^5C)$	+	$poly(c^7I)$	3000
$poly(c^7I).poly(C)$	+	MEM	600
$poly(c^7I).poly(C)$	+	poly(I)	6000
poly(I).poly(C)	+	MEM	3000 - 10000
poly(I).poly(C)	+	$poly(c^3I)$	3000 - 10000
poly(I).poly(C)	+	$poly(c^7I)$	6000
$poly(I).poly(br^5C)$	+	MEM	3000 - 10000
$poly(I).poly(br^5C)$	+	$poly(c^3I)$	3000
$poly(I).poly(br^5C)$	+	$poly(c^7I)$	6000

Footnote to Table 1

[*]   Final concentration of homopolymer duplex and triplex in reaction
     mixture : 5, 10 and 15 µg/ml MEM, respectively. The mixtures were
     incubated for 1 hour at 37° and then applied onto the cells.

[+]   Complexes refer to 1:1 or 1:2 stoichiometric mixtures of the homo-
     polymer components. The exact nature of the duplex or triplex
     formed under our experimental conditions was not investigated.

markedly inhibited complement activity in an hemolytic assay system whereas
poly(A) failed to do so (Table 2). Substitution of CH for N-3 or N-7 did
not endow poly(A) with anti-complement potency. The anti-complement behavior
of poly(I) was not affected upon substitution of CH for N-7 and was only
slightly reduced upon substitution of CH for N-3 (Table 2). When mixed with
poly(C), both poly(I) and $poly(c^7I)$ completely lost their anti-complement
activity. $Poly(c^3I)$, however, retained its anti-complement activity upon
mixing with poly(C). As would be anticipated if poly(I) and $poly(c^7I)$ added
to poly(A).poly(U) to form the triplexes poly(A).poly(U).poly(I) and
$poly(A).poly(U).poly(c^7I)$, both poly(I) and $poly(c^7I)$ lost a significant

TABLE 2. ANTI-COMPLEMENT ACTIVITY

Homopolymers or homopolymer mixtures	Hemolytic C' titers			
	Complement (diluted 1/10 in PBS) incubated for 1 h at 37° in the presence of varying polynucleotide concentrations (µg/ml) (*)			
	10	20	40	100
poly(A)	–	–	–	160
poly(c^{3}A)	–	–	–	160
poly(c^{7}A)	–	–	–	160
poly(I)	160	80	20	10-20
poly(c^{3}I)	160	160	40-80	20-40
poly(c^{7}I)	160	160	20-40	10
poly(I) + poly(C)	–	–	160	160
poly(c^{3}I) + poly(C)	–	–	80	40
poly(c^{7}I) + poly(C)	–	–	160	160
poly(A).poly(U) + poly(I)	–	–	160	80
poly(A).poly(U) + poly(c^{3}I)	–	–	80	40
poly(A).poly(U) + poly(c^{7}I)	–	–	160	160
Control PBS	160	160	160	160

(*) For homopolymer mixtures the indicated concentrations refer to each homopolymer separately.

part of their anti-complement properties when mixed with poly(A).poly(U). Poly(c^{3}I), however, fully exhibited its anti-complement activity in the presence of poly(A).poly(U) (Table 2).

Reverse transcriptase inhibition. In a standard reaction mixture employed before (8,13) to evaluate the inhibitory effects of poly(2'-azido-2'-deoxyuridylic acid) and poly(2-methyl-thioinosinic acid) on MuLV (Moloney) DNA polymerase activity, both poly(c^{7}A) and poly(c^{7}I) as well as poly(I) itself proved effective in inhibiting DNA synthesis at a concentration (circa 70 µg/ml) at which poly(A) rather stimulated [^{3}H]dTMP incorporation (Fig. 2) (7). In the same experimental conditions poly(c^{3}A) and poly(c^{3}I) showed little, if any, inhibitory effect (Fig. 2).
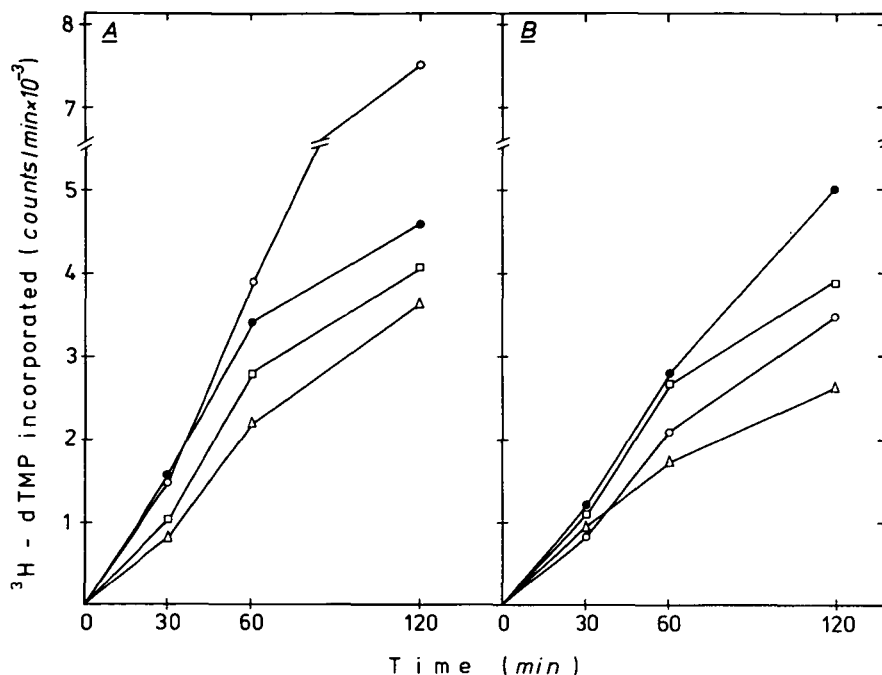
Figure 2. - Effect of poly(A), poly(c^1A), poly(c^3A), poly(c^7A), poly(I), poly(c^3I) and poly(c^7I) on DNA polymerase activity of MuLV (Moloney). DNA synthesis was measured at different times as indicated. Final concentration of polymers in assay mixture : 20 µg/280 µl.

A : ● : control. O : poly(A). □: poly(c^3A). △ : poly(c^7A).

B : ● : control. O : poly(I). □ : poly(c^3I). △ : poly(c^7I).

DISCUSSION

The data reported herein suggest a relatively greater influence of N-3 than of N-7 in the examined biologic activities of poly(I).

(1) Earlier (16), it was reported that poly(c^3A) forms both a 1:1 and 1:2 complex with poly(U). Both complexes are assumed to have a high Tm (> 80° in 0.15M Na^+, pH 7.0). Data presented herein suggest that poly(c^3I) forms a complex with poly(br^5C) (most probably of 1:1 stoichiometry) with a Tm of 52° (0.10M Na^+, pH 7.2) while it fails to form a stable complex with poly(C). Thus, while a 1:1 mixture of poly(c^3I) and poly(C) would not be expected to induce interferon, both

poly(c^3I).poly(br^5C) and poly(c^3A).poly(U) should do so, assuming that the latter does not rearrange to a triple helix under physiological conditions. Poly(c^3I).poly(br^5C) does have a lower Tm than that regarded as ideal ($\geqslant 60°$) for interferon induction; yet poly(c^7I).poly(C) which has a similar Tm [$\cong 50°$ under comparable conditions (2)] is an efficient inducer (2,3), albeit not as active as poly(I).poly(C), whereas poly(c^3I).poly(br^5C) is entirely inactive as interferon inducer. When poly(I) was mixed with either poly(c^3I).poly(C) or poly(c^3I).poly(br^5C) and when poly(c^7I) was mixed with poly(c^3I). poly(br^5C), interferon induction occurred according to displacement reactions ii-iv. These displacement reactions reflect the following order of helix stabilities :

$$poly(c^3I).poly(C) \ll poly(c^3I).poly(br^5C) < poly(I).poly(C)$$
$$< poly(c^7I).poly(br^5C) < poly(I).poly(br^5C)$$
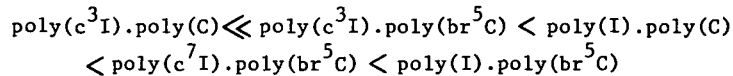
The only apparent anomaly is that poly(A) appears to react with poly(c^3A).poly(U) (equation i) to give poly(A).poly(U). This displacement reaction should not occur if poly(c^3A).poly(U) indeed has a higher Tm than poly(A).poly(U) (9). As we have established before with a large variety of polyribonucleotide complexes (16), displacement reactions are invariably directed towards the formation of the helix with the higher thermal stability.

The results obtained with mixtures of poly(c^3A).poly(U) + poly(A), poly(A).poly(U) + poly(c^3I), poly(c^3I).poly(C) + poly(I), etc. (Table 1) further indicate that there is nothing inherent about poly(c^3I) or poly(c^3A) that prevents interferon induction (e.g., cytotoxicity):

(2) Although the anti-complement activity of poly(I) analogs in which either N-7 or N-3 has been replaced by CH is not drastically altered, the relative order of decreasing activity is poly(I) $\geqslant$ poly(^{7}I) $>$ poly(c^3I). Poly(I) and poly(c^7I) lost their anti-complement potency when complexed to poly(C) (Table 2). In support of the lack of existence of a poly(c^3I).poly(C) duplex, the addition of poly(C) to poly(c^3I) failed to reduce the latter's ability to inhibit complement.

(3) Poly(c^7I), like poly(I), is also more efficacious than poly(c^3I) in inhibiting the endogenous RNA directed DNA polymerase associated with murine leukemia virus.

Poly(c^3I) differs in yet another aspect from both poly(I) and

poly(c^7I). While both poly(I) and poly(c^7I) form triple-helical complexes with poly(A).poly(U) (17 and this report) and poly(A) (12 and, e.g. 16), poly(c^3I) fails to form stable complexes with either.

In the assumption that poly(c^3A).poly(U) and poly(c^3I).poly(br^5C) maintain a double-stranded helix under physiological conditions, their failure to induce interferon may be attributed to conformational differences between these two helices and the other duplexes that are active interferon inducers. Such an hypothesis has previously been advanced (1,2, 18,19,20) to account for the observation that complexes based on poly(c^7A) or poly(L) (polylaurusin, polyformycin B) fail to induce interferon (1,18).

Apparent from the data presented is the fact that the replacement of the purine N-3 by CH significantly disturbs both the physicochemical and biological properties of poly(I). The cause of these disturbances is likely related to the increased basicity of N^1 of c^3A and the decreased acidity of N^1-H of c^3I, and/or the charge in electron distribution in both c^3I and c^3A (resulting in, e.g., altered dipole moments), and/or disturbance of the syn-anti equilibrium induced by the increased steric bulk of hydrogen with respect to a lone electron pair.

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